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Ketoprofen *S*(+) enantiomer inhibits prostaglandin production and cell growth in 3T6 fibroblast cultures

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Abstract

The ketoprofen *S*(+) enantiomer inhibits with great stereoselectivity both prostaglandin H synthase isoenzymes. Thus, the biological effects of ketoprofen on inflammation are due almost entirely to the *S*(+) isomer. Here, we report that the *S*(+) enantiomer, at doses that inhibit prostaglandin synthesis, is effective in reducing DNA synthesis and 3T6 fibroblast growth. Our data suggest that prostaglandins are involved in the control of 3T6 fibroblast growth and that the effect of the ketoprofen *S*(+) enantiomer on 3T6 proliferation is correlated with its effects on prostaglandin H synthase and prostaglandin production.

Keywords: Cell proliferation; Arachidonic acid; Eicosanoids; Growth factors; Cyclooxygenase

1. Introduction

Eicosanoids are considered to be intercellular messengers in physiopathological processes such as inflammation, but there is also strong evidence that arachidonic acid or its metabolites could play an important role in the regulation of cell proliferation. Thus, a key enzyme responsible for arachidonate mobilisation, phospholipase A_2, is activated by mitogens (Lin et al., 1993). Furthermore, cell–cell contact inhibition of 3T6 fibroblast growth is correlated with an impairment of phospholipase A_2 activity, arachidonic acid release and consequently prostaglandin E_2 formation (Lloret et al., 1996), a situation that can be reversed with a mechanical wound. Thus, prostanoids, and specifically prostaglandin E_2, play an important role in proliferation and wound repair in 3T6 fibroblast cultures (Moreno, 1997).

Two isoenzymes of prostaglandin endoperoxide H synthase, (also known as cyclooxygenase; EC 1.14.99.1.) catalyze the conversion of arachidonic acid to prostaglandins. Both isoforms are encoded by separate genes and differ in their regulation and tissue distribution. Recent evidence suggests that prostaglandin endoperoxide H synthase-2, an immediate early gene overexpressed after serum and/or growth factors, could have an important role in the control of 3T6 fibroblast growth (Martínez et al., 1997). We must consider that the chiral non-steroidal anti-inflammatory drugs inhibit with stereoselectivity both prostaglandin endoperoxide H synthase-1 and prostaglandin endoperoxide H synthase-2 isoenzymes, and the prostaglandin endoperoxide H synthase-2 inhibition observed for racemic non-steroidal anti-inflammatory drugs can be attributed almost exclusively to their *S*(+) enantiomers (Carabaza et al., 1996).

Finally, there is substantial evidence in the literature that non-steroidal anti-inflammatory drugs, which inhibit prostaglandin endoperoxide H synthase-1 and prostaglandin endoperoxide H synthase-2, can effectively reduce tumor progression in humans and experimental animals (Reddy et al., 1990; Marnett, 1992). Increased prostaglandin production by tumors has been associated with aggressive tumor progression (Honn et al., 1981) and inhibition of prostaglandin synthesis has resulted in growth retardation of experimental tumors (Lupulescu, 1978). However, other authors have reported that prostanoids are not involved in the antitumor action of non-steroidal anti-inflammatory drugs. Although these studies do not lead to a defined mechanistic pathway, alternative pharmacological properties apart from prostaglandin endoperoxide H synthase inhibition have been proposed for various non-steroidal anti-inflammatory drugs (McCraken et al., 1996). Thus, these authors demonstrated that the non-prostaglandin endoperoxide H synthase-inhibiting enantiomer of

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flurbiprofen reduced colonocyte proliferation and Chiu et al. (1997) proposed that the antitumor effect of sulindac may be independent of prostaglandin modulation.

Fibroblasts have been extensively used to analyse the mechanism of mitogenic stimulation by growth factors (Domin and Rozengurt, 1992, 1993). The effects of these factors are mediated by multiple synergistic signalling pathways, including arachidonate release and production of prostaglandin E_2 (Rozengurt, 1986). Here we present the effect of both enantiomers of ketoprofen on 3T6 fibroblast growth. Based on the dose/response relationship of *R*- and *S*-ketoprofen on fibroblast growth and prostaglandin E_2 synthesis, we propose that the effect of this non-steroidal anti-inflammatory drug on cell growth is related to prostaglandin endoperoxide H synthase inhibition and the subsequent impairment of prostaglandin formation.

2. Materials and methods

2.1. Reagents

RPM 1640, foetal calf serum, penicillin G, streptomycin, and trypsin/EDTA were purchased from GIBCO (Gaithersburg, MD). [methyl-^{3}H]thymidine (20 Ci/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA). *R*(−) and *S*(+) ketoprofen enantiomers (> 95% enantiomeric excess) were generously provided by Lab. Menarini (Badalona, Spain). Drugs were prepared as a solution in dimethylsulfoxide and, for each experiment, were freshly diluted in medium. The final concentration of dimethylsulfoxide never exceeded 0.1%. All other chemicals were reagent grade or better.

2.2. Cell culture

Murine 3T6 fibroblasts were grown and maintained as previously described (Martínez et al., 1997). Thus, cells were grown in RPMI 1640 containing 10% foetal calf serum and penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were harvested with trypsin/EDTA and passed to tissue-culture plates with a surface area of 5 cm^2/well (tissue-culture cluster 12; Costar, Cambridge, MA). Fibroblast cultures were maintained in a temperature- and humidity-controlled incubator at 37°C with 95% air–5% CO_2. Cell viability tests were performed under all experimental conditions using the trypan blue exclusion test.

2.3. Cell growth

The effect of enantiomers was assessed on 3T6 fibroblasts plated at 10^3 cells/well in 12-well plates and cultured for 3 days in RPMI 1640 supplemented with 10% foetal calf serum in the presence of different treatments. Finally, the cells were washed, trypsinized, and total cell number was assessed by counting the cells per well using a hemocytometer.

2.4. [^{3}H]Thymidine incorporation assay

DNA synthesis was measured as previously described (Martínez et al., 1997). Fibroblasts were cultured in 96-well plates (Costar) at a density of 400 cells/well. Six hours later, cells were incubated with the enantiomers and [^{3}H]thymidine (1 μCi/well) for 24 h. [^{3}H]Thymidine-containing media were aspirated, cells were overlaid with 1% Triton X-100, and then cells were scraped off the dishes and the radioactivity present in the cell fraction was measured by liquid scintillation counting.

2.5. Measurement of prostaglandin E_2 release

An aliquot of culture supernatant medium (0.25 ml) was acidified with 1 ml of 1% formic acid. Prostaglandin E_2 was extracted in ethyl acetate (5 ml), and, after the aqueous phase was discarded, the organic phase was evaporated in a stream of nitrogen. The overall recovery for the extraction procedure was established by including [^{3}H]prostaglandin E_2 and was found to be 80%. Prostaglandin E_2 levels in the medium were determined using a PGE_2-monoclonal enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI), following the manufacturer's protocol.

2.6. Data analysis

Results are expressed as mean values ± S.E.M. Differences between control and treated cultures were tested by using either the Student's *t*-test or one-way analysis of variance followed by the least significant difference test as appropriate.

3. Results

3.1. Effect of racemic ketoprofen and enantiomers of ketoprofen on growth and DNA synthesis of 3T6 fibroblasts

We have previously observed that racemic ketoprofen markedly reduces 3T6 fibroblast proliferation stimulated by the mitogenic factors of serum (Martínez et al., 1997). To determine the effect of ketoprofen enantiomers on 3T6 fibroblast proliferation, the cells were cultured in 10% foetal calf serum in the presence of drugs. Given the possible inversion of the *R*(−) to the *S*(+) enantiomer, it was necessary to change the medium every day. The proliferative response of cells was significantly reduced by the addition of racemic ketoprofen and the *S*(+) enantiomer (50–60%). This effect was dose-dependent and reached a plateau at 5 μM or 0.5 μM, respectively (Fig. 1). However, no significant antiproliferative response was seen with the *R*(−) enantiomer at doses of 5–50 μM.

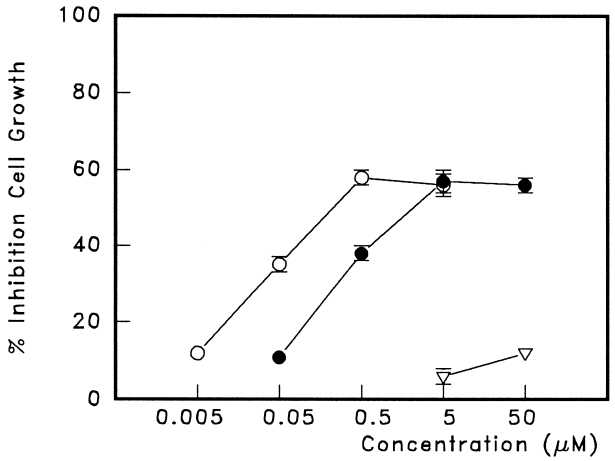

Fig. 1. Dose-dependent effect of racemic ketoprofen (●), the *S*(+) enantiomer (○) and the *R*(−) enantiomer (▽) on 3T6 fibroblast proliferation. 10^3 cells/well were plated and cultured for 3 days in RPMI 1640 with 10% foetal calf serum in the presence of drugs. Finally, cells were trypsinized and counted. The cell counting of control plate was 21,900 ± 300 cells/well. Results are mean ± S.E.M. of 3 experiments performed in triplicate.

To analyse the effect of racemic ketoprofen and enantiomers of ketoprofen on foetal calf serum-stimulated DNA synthesis, 3T6 cells were incubated in medium containing progressively increasing concentrations of drugs. As shown in Fig. 2, cells treated with racemic ketoprofen and the *S*(+) enantiomer suffered a dose-dependent impairment of [^{3}H]thymidine incorporation. Thus, racemic ketoprofen (5 μM) exhibited 50–60% inhibition of thymidine incorporation whereas the *S*(+) enantiomer produced the same inhibition at 0.5 μM. However, the *R*(−) enantiomer was not able significantly to reduce DNA synthesis stimulated by foetal calf serum. These drugs were not cytotoxic at concentrations below 50 μM and the withdrawal of the treatments was associated with the return of the original cell proliferation capacity (data not shown).

3.2. Effect of prostaglandins on the antiproliferative effect of racemic ketoprofen and the S(+) enantiomer

In Swiss 3T6 cells stimulated by foetal calf serum, the major arachidonic acid metabolite is prostaglandin E_2 (Lloret et al., 1996), a product of the prostaglandin endoperoxide H synthase pathway. Moreover, products of the cycloxygenase pathway have been implicated in the control of growth and functioning of fibroblasts in culture (Lloret et al., 1996; Moreno, 1997). Given these facts, the synthesis of prostaglandin E_2 in the presence of the enantiomers of ketoprofen was studied. As we can see in Fig. 3, racemic ketoprofen and the *S*(+) enantiomer induced a dose-dependent inhibition of prostaglandin E_2 synthesis, with a maximum effect at 5 μM, whereas the *R*(−) only had an appreciable effect at 50 μM, which may be attributed to contamination with the *S*(+) enantiomer (approximately 0.5%).

To confirm whether inhibition of prostaglandin E_2 production is implicated in the antimitogenic effect of racemic and *S*(+) ketoprofen, 3T6 fibroblasts were incubated in medium containing these drugs plus exogenous prostaglandin E_2. The results presented in Fig. 3 together with previous results (Moreno, 1997) show that foetal calf serum (10%) produced about 100–200 pg/ml of PGE_2 per 1000 cells. Considering these findings, we observed

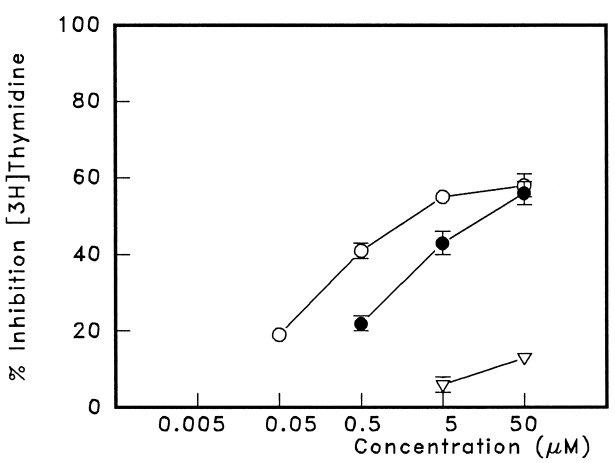

Fig. 2. Dose–response curve for the inhibition of DNA synthesis by racemic enantiomer (●), the *S*(+) enantiomer (○) and the *R*(−) enantiomer (▽). Cultures of 3T6 were incubated in medium containing 10% foetal calf serum in the presence or absence of drugs and [^{3}H] thymidine incorporation was assessed as described. The [^{3}H] thymidine incorporation induced by 10% foetal calf serum was 405 ± 11 cpm/1000 cells. The results are expressed as the percentage of inhibition and are the mean ± S.E.M. of nine measurements from three independent experiments.

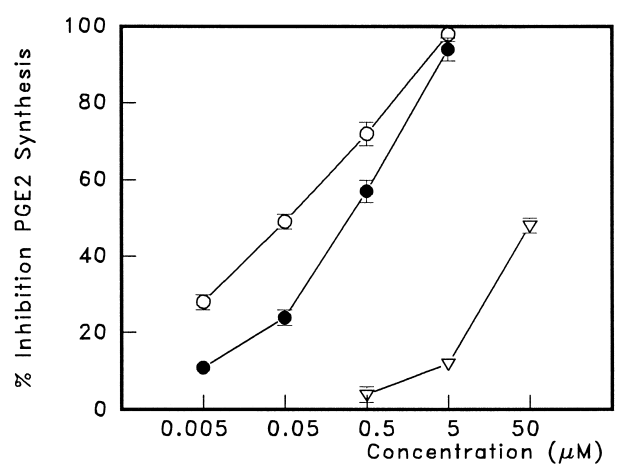

Fig. 3. Dose-dependent effect of racemic ketoprofen (●), the *S*(+) enantiomer (○) and the *R*(−) enantiomer (▽) on prostaglandin E_2 synthesis by 3T6 fibroblasts. Subconfluent 3T6 cultures were incubated in RPMI 1640 with 10% foetal calf serum in the presence or absence of drugs for 24 h at 37°C. Finally, prostaglandin E_2 levels in the culture medium were measured. The prostaglandin E_2 concentration in culture supernatant in control conditions was 126 ± 7 pg/1000 cells. Results are means ± S.E.M. of 3 experiments performed in triplicate.

Table 1
Effect of treatments on cell growth and DNA synthesis were effected by the prostaglandin E_2 levels

	Cell growth ($\times 10^3$ cells/well)	DNA synthesis (Dpm $\times 10^3$)
Control	58 ± 3	162 ± 9
Racemic	24 ± 2^a	93 ± 5^a
Racemic + prostaglandin E_2	49 ± 2^b	146 ± 6^b
$S(+)$ enantiomer	23 ± 2^a	89 ± 4^a
$S(+)$ enantiomer + prostaglandin E_2	48 ± 2^b	147 ± 5^b

3T6 fibroblasts (10^3 cells/well) were incubated in RPMI 1640 supplemented with 10% foetal calf serum and racemic ketoprofen (5 μM) or the $S(+)$ enantiomer (0.5 μM) in the presence or absence of prostaglandin E_2 (100 pg/ml). Finally, cell growth and DNA synthesis were determined as described in the materials and methods. Values are means ± S.E.M. of 5–6 measurements. [a] $P < 0.05$, significantly different from control cells. [b] $P < 0.05$, significantly different from treated cells without prostaglandin E_2.

that the addition of prostaglandin E_2 (100 pg/ml) counteracts the inhibitory effect of drugs active on prostaglandin endoperoxide H synthase, racemic ketoprofen and the $S(+)$ enantiomer (Table 1).

4. Discussion

Although several lines of evidence demonstrate an inverse relationship between the use of non-steroidal anti-inflammatory drugs and proliferative processes such as intestinal cancer, Chiu et al. (1997) proposed that this effect may be independent of prostaglandin modulation.

The objectives of this study were to evaluate the effect of both enantiomers of ketoprofen on growth and DNA synthesis in 3T6 fibroblast cultures and to determine whether alterations in prostaglandin biosynthesis are related to the effect of these drugs on cell proliferation.

Ketoprofen, a propionic acid with analgesic and anti-inflammatory activities, has an asymmetric carbon atom, exhibiting optical isomerism. We have previously observed that its biological activity is due almost entirely to the $S(+)$ enantiomer (Moreno et al., 1990). Moreover, Carabaza et al. (1996) reported recently that the $S(+)$ enantiomer of ketoprofen inhibits with great stereoselectivity both prostaglandin H synthase isoenzymes.

Our results show that the prostaglandin endoperoxide H synthase-inhibiting molecules, racemic ketoprofen and the $S(+)$ enantiomer were effective in reducing DNA synthesis and cell growth in 3T6 fibroblast cultures at doses that inhibit prostaglandin synthesis. On the other hand, the $R(-)$ enantiomer, a non-prostaglandin endoperoxide H synthase-inhibiting compound, was ineffective. In agreement with our previous results, these data are consistent with the hypothesis that eicosanoids could be involved in the regulation of physiological and pathophysiological cell proliferation (Moreno, 1998). Interestingly, Tsujii et al. (1997) have recently observed that cyclooxygenase-2 transfected human colon cancer cells (Caco-2) exhibit an enhancement of prostaglandin E_2 synthesis, growth and invasiveness. This increased invasiveness and prostaglandin production was reverted by treatment with sulindac sulfite, a prostaglandin H synthase inhibitor like ketoprofen.

In summary, our data suggest that prostaglandins are involved in the regulatory processes of 3T6 fibroblast growth and that the effect of ketoprofen on 3T6 proliferation is correlated with its effect on prostaglandin H synthase and prostaglandin production.

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